Freezing and Calcium Chloride Marination Effects on Beef Tenderness and Calpastatin Activity[1,2]

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ABSTRACT: Because freezing samples decreases calpastatin activity and the application of exogenous calcium activates the calpain proteolytic system, thereby improving tenderness, the objective of this study was to determine whether freezing would enhance the effects of $CaCl_2$ marination on the tenderness of beef steaks. Longissimus steaks were obtained from 10 beef steers 6 d postmortem. One-half of the steaks were frozen at −30°C for 6 wk. The remaining steaks were treated fresh; one-half were subjected to a 150 m*M* $CaCl_2$ marinade for 48 h. Frozen steaks were thawed and subjected to the same treatment. Treatments consisted of 1) fresh control, 2) fresh marinated, 3) frozen control, and 4) frozen marinated. Samples were taken before and after treatment (6 and 8 d) for calpastatin activity determination and d 8 for SDS-PAGE. Warner-Bratzler shear force values were measured 8 d postmortem. Data were analyzed using a paired comparison *t*-test procedure. Results showed that freezing and marination significantly decreased calpastatin activity. A .35-kg improvement (P = .07) in Warner-Bratzler shear force was observed with freezing, whereas a .78-kg improvement (P < .01) in tenderness was observed with marination. However, prior freezing enhanced the effects of marination. Therefore, the decrease in calpastatin activity seemed to allow greater proteolysis by the calpains with the application of Ca^{2+}. The SDS-PAGE of myofibril preparations indicated that more small polypeptide fragments (28 to 32 kDa) appeared and a 95-kDa fragment was more intense in the marinated samples than in control samples, indicating that proteolysis was enhanced. In conclusion, tenderness was improved by $CaCl_2$ marination and prior freezing intensified its effect.

Key Words: Tenderness, Calpastatin, Beef, Freezing, Calcium

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Introduction

The application of Ca^{2+} to improve tenderness is well documented whether applied prerigor (Koohmaraie et al., 1988, 1989, 1990; Koohmaraie and Shackelford, 1991; Morgan et al., 1991; Wheeler et al., 1991) or postrigor (Alarcon-Rojo and Dransfield, 1989; Whipple and Koohmaraie, 1992). This improvement in tenderness is thought to occur mainly through the activation of the calpain (Ca^{2+}-dependent) proteolytic system that hydrolyzes key structural myofibrillar proteins during postmortem aging. Although μ-calpain is normally active postmortem, introducing millimolar concentrations of exogenous Ca^{2+} activates m-calpain. Both μ- and m-calpain hydrolyze the same myofibrillar proteins (Goll et al., 1989), and *in vitro* studies show that myofibrils incubated with either μ- or m-calpain closely mimic proteolysis that occurs during the tenderization of meat (for review see Goll et al., 1983; Koohmaraie, 1988). However, both proteinases are inhibited by calpastatin, a specific endogenous inhibitor. Whipple et al. (1990) reported a significant relationship between calpastatin 24-h activity and the amount of postmortem proteolysis associated with tenderness. Therefore, the endogenous calpastatin activity may partially hinder proteolysis by the calpains when exogenous Ca^{2+} is applied. Because calpastatin is

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[2]Mention of a trade name, proprietary product, or specific equipment is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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susceptible to inactivation by freezing (Koohmaraie, 1990), the objective of this study was to determine whether prior freezing of meat would enhance $CaCl_2$ marination effects on tenderness.

Materials and Methods

Four longissimus steaks, 2.54 cm thick, were obtained 6 d postmortem from each of 10 beef steers approximately 15 mo of age. Before treatment, a 5-g sample was obtained to determine calpastatin activity. Treatments included 1) fresh control, 2) fresh marinated, 3) frozen control, and 4) frozen marinated. One steak from each animal was marinated for 48 h in 600 mL of cold 150 m*M* $CaCl_2$ at 4°C. The other steak served as the nonmarinated control, which was vacuum-packaged and stored at 4°C for the same duration. After marination was completed, a 6-g sample was removed. From this sample, 1 g was used for SDS-PAGE, and 5 g was used for determining calpastatin activity. The other two steaks from each steer were frozen at –30°C for 6 wk, after which steaks were thawed for 18 h at 4°C. Thawed steaks were then treated as previously outlined for the fresh steaks. Upon completion of each treatment, steaks were immediately cooked on Farberware Open-Hearth Broilers (Farberware, Bronx, NY) to an end-point temperature of 70°C monitored with iron constantan thermocouple wires. After cooking, steaks were tempered at 4°C for 24 h. Six 1.27-cm-diameter cores were removed from each steak parallel to muscle fiber direction. Instron Warner-Bratzler shear values then were determined on each core and an average was calculated.

For calpastatin activity determination, 5-g samples were extracted in 150 m*M* Tris-HCl, pH 8.3, 50 m*M* ethyleneglycol N,N,N′,N′-tetraacetic acid (EGTA), and 7 m*M* 2-mercaptoethanol (MCE). Homogenates were centrifuged at 30,000 × g_{max} for 1 h and supernatants were filtered, pH adjusted to 7.3, and dialyzed against 20 m*M* Tris-HCl, pH 7.35, .5 m*M* EDTA, and 7 m*M* MCE. These procedures were conducted at 4°C. After dialysis, supernatants were heated at 95°C for 10 min in 6-mL aliquots, cooled on ice, and then centrifuged at 2,500 × g_{max} for 30 min. Supernatants were retained and centrifuged at 30,000 × g_{max} for 1 h. Volumes were determined and assay procedures of Koohmaraie (1990) were followed, except that up to 1 mL of each sample was added to 1 mL of casein media with 5 m*M* $CaCl_2$ in the assay. Total assay volume was 2.12 mL. One unit of calpastatin activity was defined as the amount of calpastatin that inhibited one unit of m-calpain activity.

Muscle samples for SDS-PAGE were homogenized in 50 m*M* EGTA, 100 m*M* Tris, 60 m*M* K_2HPO_4, 100 m*M* KCl, 1 m*M* $MgCl_2$, 1 m*M* NaN_3, pH 7.0, using a Polytron (Brinkmann Instruments, Westbury, NY) at half speed for 10 s. Homogenates were centrifuged at 1,000 × *g* for 10 min. The myofibril pellet was subjected to four more washes in 20 m*M* potassium phosphate, 100 m*M* KCl, 1 m*M* EGTA, 1 m*M* $MgCl_2$, and 1 m*M* NaN_3, pH 7.0. Samples then were prepared for SDS-PAGE by dissolving them in 62.5 m*M* Tris-HCl (pH 6.8), 10% glycerol, 5% MCE, 2% SDS, and .02% bromophenol blue and boiling for 5 min. Electrophoretic procedures of Laemmli (1970) were followed. Proteins were separated (80 μg) using a discontinuous 7.5 to 15% acrylamide gradient slab gel with a 75:1 acrylamide:bisacrylamide ratio. Protein concentrations were determined using the biuret procedure (Gornall et al., 1949) with BSA as the standard.

Data were analyzed using a paired comparison *t*-test procedure (SAS, 1985) of the hypothesis that the mean difference of two groups are equal to zero.

Results and Discussion

In a previous study, we found that marinating beef steaks at 5 d postmortem in $CaCl_2$ improved tenderness (Whipple and Koohmaraie, 1992). Yet, in that study, longissimus steaks from steers fed a β-adrenergic agonist remained less tender than the control steaks even after marination. A possible explanation was that the higher calpastatin activity partially prevented proteolysis by the calpains, because feeding a β-adrenergic agonist significantly increases calpastatin activity (Kretchmar et al., 1989; Koohmaraie and Shackelford, 1991; Koohmaraie et al., 1991). Also, 24-h calpastatin activity is inversely related to the degree of postmortem proteolysis (Whipple et al., 1990). Thus, if calpastatin activity could be decreased, proteolysis by calpain would be enhanced regardless of treatment, diet, age, and so on. Because calpastatin is susceptible to freezing (Koohmaraie, 1990), our objective was to determine whether freezing before $CaCl_2$ marination would improve its effect on tenderness via decreasing the ability of calpastatin to inhibit proteolysis by the calpains.

As expected, calpastatin activity was significantly reduced with freezing (Table 1) in the d-6 samples and d-8 nonmarinated samples. However, there was no difference ($P > .05$) in calpastatin activity between the fresh and frozen marinated steaks. Yet, marination exerted a greater effect on calpastatin activity in the fresh steaks (1.41 units/g difference) than in the previously frozen steaks (.97 units/g difference), and that difference (1.41 – .97 = .44 units) corresponded to the effect of

Table 1. Means difference and SE for calpastatin activity as affected by freezing, day postmortem, and $CaCl_2$ marination[a]

Item	Means difference[b]	SE	Variance	P-value
Fresh d 6 – frozen d 6	.32	.11	.11	.02
Fresh d 8 – frozen d 8, NM	.45	.20	.36	.05
Fresh d 8 – frozen d 8, M	–.02	.19	.31	.93
d 6 – d 8, Fresh	.02	.15	.21	.91
d 6 – d 8, Frozen	.06	.14	.18	.66
NM d 8 – M d 8, Fresh	1.41	.09	.07	<.01
NM d 8 – M d 8, Frozen	.97	.13	.17	<.01
(NM; fresh + frozen) – (M; fresh + frozen)	1.17	.10	.08	<.01

[a]NM = nonmarinated and M = marinated.
[b]The amount of calpastatin that inhibits one unit of m-calpain activity/gram of muscle.

freezing on calpastatin activity (.45 units/g). Earlier, we found that both calpain and calpastatin activities declined with $CaCl_2$ marination, and the loss of calpastatin activity was attributed to its being hydrolyzed by m-calpain (Whipple and Koohmaraie, 1992), because calpastatin can serve as a substrate for calpains (Mellgren et al., 1986).

Tenderness as determined by Warner-Bratzler shear force tended (P = .07) to improve (decrease in shear force values) with freezing for the nonmarinated steaks (Table 2). Winger and Fennema (1976) and Crouse and Koohmaraie (1990) showed that if beef muscle is frozen at 1 d postmortem, thawed, and then aged, tenderness is improved. It was suggested by Crouse and Koohmaraie (1990) that this improved tenderness may be due to increased proteolysis by calpains, because calpastatin is not as active in previously frozen muscle, whereas calpains remain fully active (Koohmaraie, 1990). Thus, the potential of freezing then aging meat to improve tenderness exists. However, μ-calpain loses activity during postmortem storage (Koohmaraie, 1988). Therefore, to maximize the freezing effect on meat tenderness that is mediated by the calpain proteolytic system, meat should be frozen as soon as rigor is complete; by doing so maximum μ-calpain activity will be preserved because μ-calpain is not susceptible to freezing. However, when steaks are cooked soon after thawing, effects of freezing on tenderness are often inconsistent (Smith et al., 1968; Berry et al., 1971; Kemp et al., 1976; Jeremiah, 1980; Wheeler et al., 1990), which likely is due to differences in freezing temperature and rates (Hankins and Hiner, 1940; Hiner et al., 1945). Histological examination by Hiner et al. (1945) revealed that the increase in tenderness was proportional to the intrafibrillar ice formation and was due to a combination of rupture of muscle fibers and rupture and stretching of connective tissue. Therefore, freezing temperature and rate as well as thaw rate may affect the extent to which aging meat after freezing improves tenderness, because of possible detrimental or beneficial effects of freezing itself.

Whether steaks were fresh or frozen, tenderness was improved with $CaCl_2$ marination, which agrees with the improvement in tenderness observed with $CaCl_2$ application at least 24 h postmortem by Alarcon-Rojo and Dransfield (1989) and Whipple and Koohmaraie (1992). Freezing improved (P < .01) the effectiveness of marination as indicated by lower shear force values compared with both fresh, marinated steaks and frozen,

Table 2. Means difference and SE for Warner-Bratzler shear force as affected by freezing and $CaCl_2$ marination[a]

Item	Means difference, kg	SE	Variance	P-value
Fresh – frozen, NM	.35	.17	.26	.07
Fresh – frozen, M	.86	.08	.06	<.01
NM – M, Fresh	.78	.17	.27	<.01
NM – M, Frozen	1.36	.14	.21	<.01
(NM; fresh + frozen) – (M; fresh + frozen)	1.03	.13	.16	<.01

[a]NM = nonmarinated and M = marinated.

nonmarinated steaks. Therefore, improvement in tenderness by $CaCl_2$ marination apparently derives from increased calpain proteolysis, because the addition of exogenous Ca^{2+} activates the calpain present, which is reflected by a decrease in calpastatin activity (Koohmaraie et al., 1988, 1989, 1990; Whipple and Koohmaraie, 1992). In addition, calpastatin activity decreased with freezing, which enhanced the effects of marination on tenderness. It is also possible that freezing ruptured cell membranes, allowing more Ca^{2+} to enter the muscle cell.

Only minor differences indicating further proteolysis appeared through SDS-PAGE (Figure 1), and there were no apparent differences between fresh and frozen samples. Because the hydrolysis of key myofibrillar proteins (i.e., desmin and troponin-T) had already occurred by 8 d postmortem in the control steaks, only subtle SDS-PAGE differences would be expected. A polypeptide of approximately 95 kDa was more intense, and more small fragments of 27 to 32 kDa appeared, which indicates that proteolysis was enhanced to some degree. The disappearance of desmin and the appearance of 94- and 28- to 32-kDa polypeptides were reported with calpain activation by $CaCl_2$ infusion into lamb carcasses (Koohmaraie and Shackelford, 1991). In this study, undoubtedly some protein solubilization occurred, but the contribution of these proteins to tenderness is unknown, especially because the proteins solubilized with increases in ionic strength are not those associated with stability of myofibrils (Wu and Smith, 1987; Taylor and Etherington, 1991).

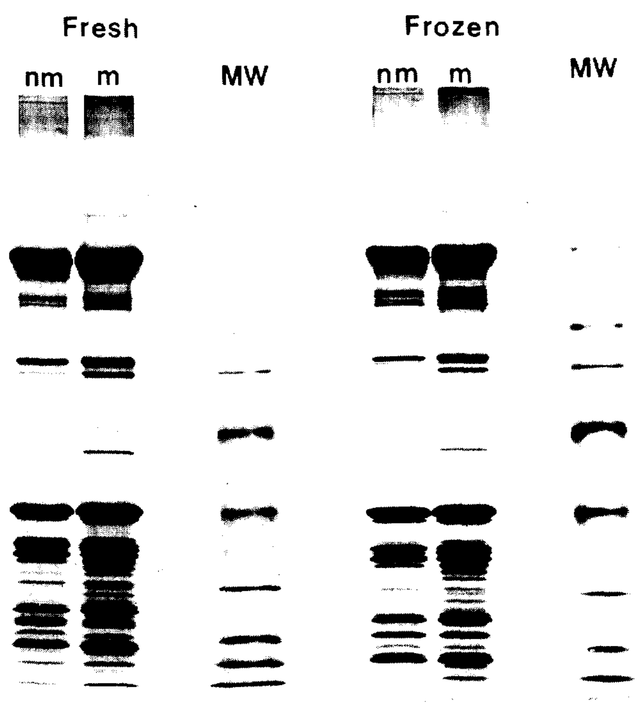

Figure 1. The SDS-PAGE (7.5 to 15%) of myofibril preparations from fresh and frozen longissimus steaks 8 d postmortem either marinated (m) or nonmarinated (nm). Molecular weight standards (MW) are, from top to bottom, as follows: myosin, 200 kDa; *E. coli* β-galactosidase, 116.3 kDa; rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg-white ovalbumin, 45 kDa; bovine carbonic anhydrase; 31 kDa; soybean trypsin inhibitor, 21.5 kDa; and hen egg-white lysozyme, 14.4 kDa.

Implications

Calcium-containing marinades are successful in improving beef tenderness by activating the calpain proteolytic system. Prior freezing enhances the ability of the system to improve tenderness by decreasing the activity of calpastatin, which is a specific inhibitor of the calpains. Normal household or restaurant use of calcium-containing marinades is feasible, and prior freezing, which often occurs, would enhance its tenderization effect.

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